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Quantitative Fluorescein Angiography Following Diode Laser Retinal Photocoagulation

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Objective: **An in vivo study was done to establish if laser-induced damage of the retina could be quantified using fluorescein angiography.**
Method: **This study was carried out on rabbit eyes (n = 6) with an 810 nm diode laser (spot diameter: 500 μm, pulse duration: 1 second, power: 100 mW–400 mW) adapted on a slit lamp. Fluorescence measurements were performed with a fundus camera connected to a fluorescence imaging system. Fluorescence staining of the retina was evaluated by mathematical modeling. Lesions were correlated to laser parameters and to histologic data.**
Results: **Image analysis shows that the laser lesions stained progressively. Fluorescence appears first at the borders of the lesion exhibiting a fluorescent ring. A progressive increase of the fluorescence into the central zone is observed. The maximum fluorescence intensity into the center of the laser spot is obtained after a delay depending on the laser energy. Below 100 ± 20 mW, lesions are detectable by fluorescence imaging only. A fluorescence plateau appears for a threshold light dose above 200 ± 20 mW. Mathematical modeling demonstrates that quantitative assessment of laser-induced damage to the retina is feasible using fluorescence imaging.**
Conclusion: **The quantification of fluorescence staining in terms of both intensity and time can contribute to a better quantification of laser-induced damage. At last, since laser damage may mimic naturally occurring pathology, this method should also be considered to quantify different types of lesions. Lasers Surg. Med. 24:338–345, 1999.**

Key words: fluorescein angiography; photocoagulation; laser; image analysis

INTRODUCTION

There is an increasing interest in the clinical application of diode lasers. Evidence of their usefulness for the treatment of choroidal conditions has been demonstrated by studies showing the efficacy of diode laser photocoagulation in the treatment of choroidal neovascular membranes or diabetic macular edema [1,2]. However some doubts have been raised as to the suitability of the infrared lasers for retinal photocoagulation because of difficulties for the control of the intensity of the retinal burn [3]. For central retinal pathologies, the best preservation of central visual function would require the most adequate dose of laser

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irradiation. However, a number of efforts to optimize the dosage of laser induced photocoagulation has had limited or no success. Currently the usual method for ophthalmologists to control the laser-induced thermal damage remains the bare observation of blanching of the retina after a laser exposure. Since fluorescein angiography is commonly used by ophthalmologists, this technique was already proposed in the mid-sixties to study laser-induced retinal lesions [4]. Several authors have also proposed to use sodium fluorescein (NaF) to study different type of lesions [5–11]. NaF displays several advantages compared to other fluorescent dyes [12]. NaF is a lipophilic dye and its partition coefficient is 4.9 at pH 7.38. For example, the lipid solubility of fluorescein is 1,000 times lower for carboxyfluorescein than for NaF. The protein binding of these dyes does not influence the penetration of the dye through cell membranes [13]. This can explain its ability to penetrate more easily the blood ocular barrier layers after IV injection. Most of the studies dedicated to NaF fluorescence kinetics described the residence time of fluorescence during angiography after NaF intravenous administration. Data concerning kinetics of fluorescence of the retinal tissue are limited to the study of undamaged tissue. In that case, it was shown that after bolus administration, fluorescein may be cleared from retinal tissue and follows a conventional bi-exponential decay using a bi-compartmental model [14]. In our study, the ability of the dye to diffuse through the damaged tissue is a key property in order to visualize the damaged area. Thanks to fluorescence imaging systems using video-image processing, quantitative assessment of the laser-induced damage could represent a valuable application of fluorescein angiography. The present in vivo study was performed on rabbit eyes with an 810 nm diode laser. It aimed to establish the role of fluorescein angiography in the quantification of laser-induced damage in ophthalmology.

MATERIALS AND METHODS

Figure 1a shows a schematic drawing of the experimental setup.

Diode Laser

An 810 nm diode laser, OcuLight®, IRIS Medical Instruments Inc. (Mountain View, CA, USA) was adapted on a slit lamp (TAKAGI, Tokyo, Japan). This diode laser system was used with a three mirror Goldman lens in a continuous mode (Fig. 1b). In order to increase the reproducibility of the laser irradiation, the spot diameter on the retina and the pulse duration were kept constant (spot diameter: 500 μm, pulse duration: 1 second). The laser power ranged from 100 mW to 400 mW. The power was verified with a PW-2 power meter (Synrad, Bothell, WA, USA). The fluence on the retina ranged from 50 W/cm^2 to 210 W/cm^2.

Animals

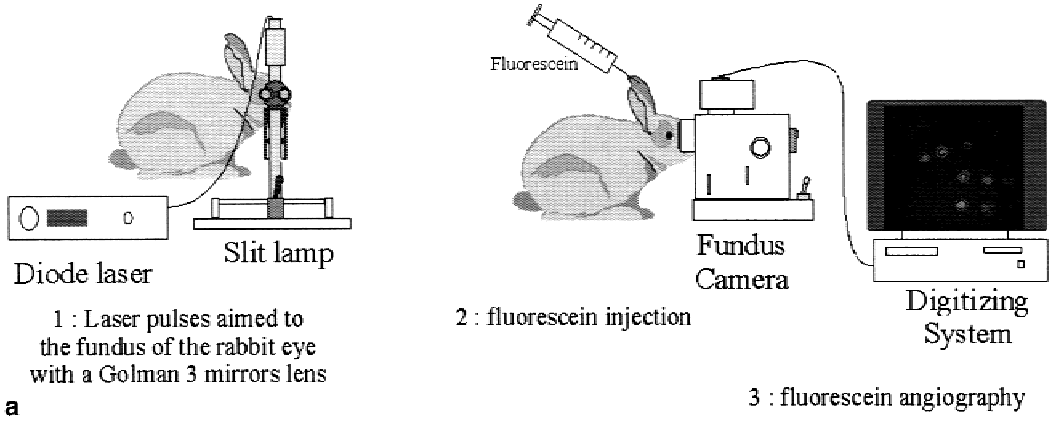
The rabbit eye was used in this study as a healthy eye model for photocoagulation without any opacities or heterogeneity of the lens. The laser pulses were performed on the mid periphery on the retina since the homogeneous pigmentation of the fundus reduced the variation of laser light absorption. We used three pigmented male rabbits weighing 3.0–3.5 Kg each. The animals were treated according to the ARVO statement for the Use of Animals in Ophthalmology and Vision Research. The rabbits were first slightly anesthetized with an intramuscular injection of a combination of Ketamine (Rhône Mérieux, France) (150 mg/Kg) and Chlorpromazine (Spécia Rhône Poulenc Rorer, France) (0.50 mg/Kg). Ketamine and Chlorpromazine were then injected into the marginal ear vein to ensure the anesthesia of the animal during the whole experimentation. Pupil dilatation was achieved with Tropicainamide 0.5% (MSD-Chibret, France) and Phenylnephrine (10%) (MSD-Chibret, France) eye drops. After completing the experiments, the animals were killed with an overdose of Ketamine and Chlorpromazine.

Fluorescence Imaging System

A CF-60UVi Canon-Europe fundus camera (Amstelveen, The Netherlands) was coupled to a Kodak Megaplus 1,317 × 1,035 pixels, 8 bits monochrome camera. The output of the camera was fed to a digitizing system OcuLab, Life Science Resources Ltd., (Cambridge, England) using a personal computer. The fundus camera was used in a 60° field mode with excitation and emission filters set for the angiography mode. The excitation flash intensity was selected in order to avoid any saturation when fluorescence intensity was maximum (F-3 corresponding to Iso sensitivity ISO = 100).

Methods

After anesthesia, 6–10 laser impacts were delivered to the mid peripheral region of the



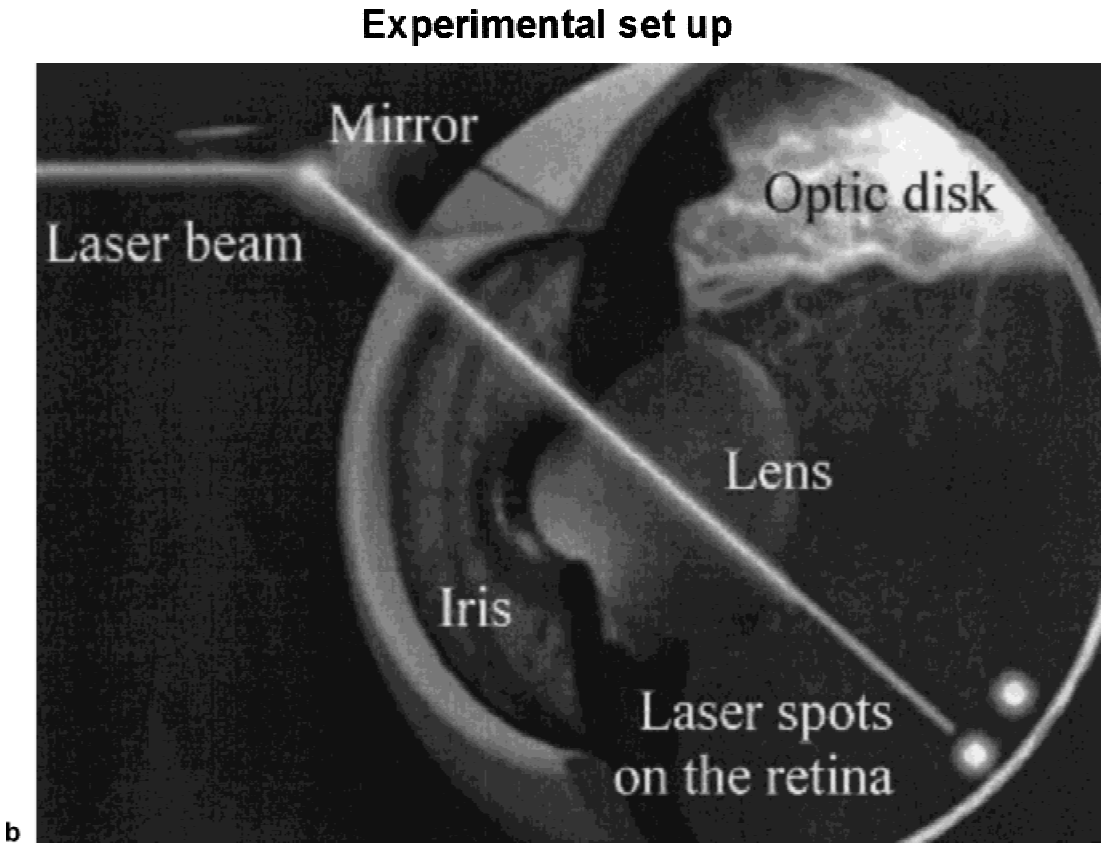


Fig. 1. **a:** Schematic drawing of the experimental setup. **b:** Schematic drawing of the eye showing the position of the Goldmann lens and the intra-ocular path of the laser beam was added.

retina inferior to the myelinated fibers layer, one disk diameter apart, on each eye (n = 6). Sodium Fluorescein (SERB Paris, France) was injected 30 minutes after the laser irradiation. A 10% solution, corresponding to 10 mg/kg body weight was injected within 2 seconds into the marginal ear vein. Two fluorescence images of the retina were recorded before sodium fluorescein (NaF) injection in order to evaluate the electronic noise of the camera. After the sodium fluorescein injection, images were recorded every 10 seconds in the first 2 minutes, then every minute for up to 12 minutes. During the acquisition of these images the fluorescence spots were usually visible with the

continuous lamp illumination between the flashes. The flash intensity was kept constant to quantify the fluorescence intensity as a function of time. The images were focused on the fluorescent spots to limit optical aberration that could occur during spot size measurements near the edges of the images.

Analysis of the Images

The fluorescence images were stored on optical disk. Their analysis provided information about the size and the shape of the fluorescence spot and the fluorescence intensity. The size of the fluorescence spots (n = 48) and the evolution of the fluorescence intensity were analyzed as a function of the light dose. The fluorescence was quantified at the edges and at the center of the laser spots.

Mathematical Modeling

Kinetics of the fluorescence intensity of the center of the laser spot and of the surrounding undamaged tissue was particularly considered in this study. Mathematical modeling based on that already used by Grotte was applied [14]. In this model used for undamaged tissue, NaF is assumed to be distributed between two compartments. Consequently, for studying undamaged tissue, one can assume that two processes coexist. The first one is the NaF diffusion process toward the center as a function of time. This diffusion process from the edges to the center can be approximated by a monoexponential function (Term A) appropriate to an open one compartment pharmacokinetic model. This model includes the following assumptions: 1) fluorescein emission is considered to be stable, is not lost, and it is not degraded or bound to receptor, 2) periphery is a continuous source of fluorescein and does not decrease during the studied period of time.

$$I_{center} = I_{max} (1 - e^{-\alpha t}) \quad (A)$$

I_{center} is the fluorescence intensity at the center of the laser spot, I_{max} is the maximum fluorescence intensity (arbitrary unit) at the center of the laser spot, and α (s^{-1}) represents the time constant of the infilling of the central zone.

The second term (B) is that the equation exists only if the tissue remains undamaged wherein a NaF tissue wash out can take place. So, NaF can be cleared out of the undamaged tissue and following similar assumptions, the fluorescence intensity can also be approximated by a monoexponential function (Term B).

$$I_{center} = Ce^{\beta t} \quad (B)$$

β (s^{-1}) represents the time constant of the clearance process, C is a fitting parameter.

In order to determine the fluorescence intensity at the center of the laser spot, the following equation can be applied.

$$I_{center} = I_{max} [(1 - e^{-\alpha t})] - Ce^{\beta t} \quad (1)$$

However, if the tissue is irreversibly damaged, the wash out process does not exist. The term (B) is not considered and the equation is restricted to term (A):

$$I_{center} = I_{max} (1 - e^{-\alpha t}) \quad (2)$$

Histology

The histological analysis was performed in order to compare the extent of retinal thermal damage in laser irradiated spots to the fluorescence appearance and fluorescence intensity evolution recorded in these damaged spots. Both eyes of each animal were enucleated and prepared for standard histology. Each eye was sectioned and the area of interest was fixed, dehydrated and embedded in paraffin. Sections of 6 μm were cut using a microtome, then counterstained with Masson trichrome. The tissue was examined with an Axiophot microscope (Zeiss, Germany).

RESULTS

The angiographic study includes the evaluation of background fluorescence and fluorescence of photocoagulated areas at the center and the borders of the irradiated spots. For all recordings, the background fluorescence increases almost immediately after injection to reach its maximum at 20 seconds after injection. This fluorescence decreases very rapidly and reaches 50% of its maximum value at 1 minute and 20% at 2 minutes. The fluorescence reaches the initial fluorescence background after 10 minutes.

The fluorescence at the center of the laser spot depends greatly on laser dosages. Figure 2 shows laser impacts on the retina obtained for different dosages (1:250 mW, 2:300 mW, 3:200 mW, and 4:100 mW) for three different delays af-

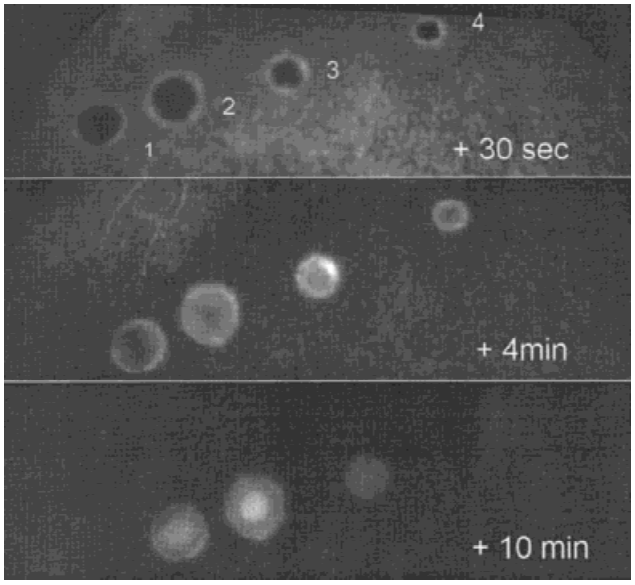


Fig. 2. Fundus fluorescent images obtained 30 seconds, 4 minutes, and 10 minutes after Sodium Fluorescein injection. Powers are the following: **#1:** 200 mW, **#2:** 250 mW, **#3:** 150 mW, **#4:** 100 mW (810 nm diode laser, P = 100–400 mW, (Circle with line = 500 μm, 1 second).

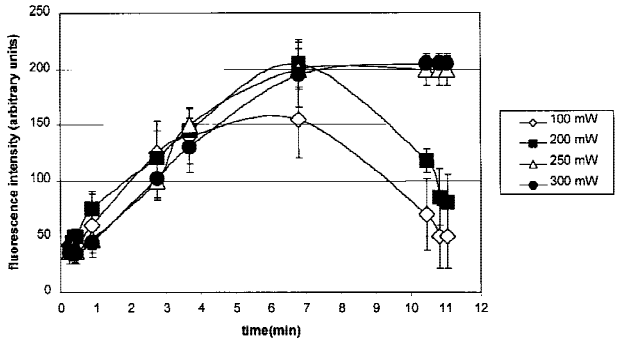


Fig. 3. Fluorescence intensity curves recorded at the center of the laser spots for the four different fluence rates: 100, 200, 250, and 300 mW. Each point is the average of five values recorded on different laser spots.

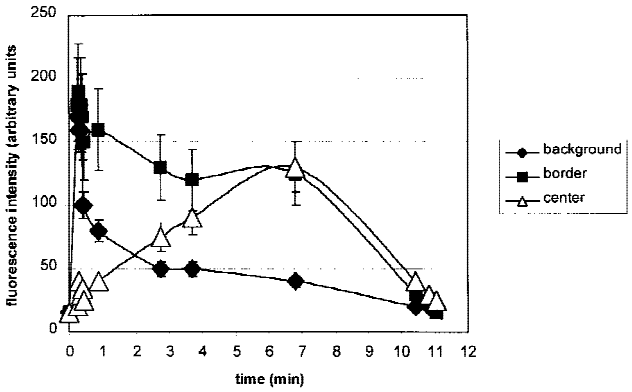


Fig. 4. Fluorescence intensity as a function of time recorded at the center and at the periphery of the laser spot #4 (100 mW). Background fluorescence is also traced. This figure shows clearly that after 6 minutes, the fluorescence decrease at the borders is almost similar to that recorded at the center, giving after a few minutes a homogeneous fluorescence spot.

ter NaF injection: 30 seconds, 4 minutes, and 10 minutes. Figure 3 shows the evolution of the fluorescence intensity at the center of the laser spot.

Thirty seconds after injection, the laser spots show a dark center indicating a very low fluorescence surrounded by a fluorescent ring. The center of the spot is even darker than the background fluorescence. At 4 minutes, the fluorescence of the center and of the borders of the laser spots are almost similar. At 10 minutes, the fluorescence of the laser spot depends greatly on the energy dosage. For spots #3 and #4 (power below 200 mW), the fluorescence is homogeneous in the laser spot. Fluorescence of spot #4 is very low, almost not detectable on the picture. For spots #1 and #2, (powers equal to 250 mW and 300 mW), an intense fluorescence is observed at the center, brighter than that observed at 4 minutes.

Figure 4 corresponding to spot #4 (100 mW), shows the evolution of fluorescence intensity as a function of time. The curves are displayed: at the center of the laser spot, at the edges, and background fluorescence is also traced. This figure shows clearly that after 6 minutes, the fluorescence decrease at the borders is almost similar to that recorded at the center, giving after a few minutes a homogeneous fluorescence spot. For this particular case, at 10 minutes, the fluorescence intensity of both center and borders is very low, almost comparable to the background fluorescence intensity.

Figure 5 shows the experimental fluorescence intensity curves for 100 mW and 300 mW (filled squares). A fit function according to equation 1 was used to mathematically describe the experimental data (solid line).

Two cases were considered:

In the first one, power is equal to 100 ± 20 mW. In this case, the tissue is altered: Histology shows that vascular damages are observed such as dilated lumens in veins, arteries, and capillaries. However, when using 100 ± 20 mW, the choroid remains undamaged and the retina and sclera display a quite normal aspect. For mathematical modeling, both term A & term B of equation 1 are used. The following parameters are used: $\alpha = 0.2\ (s^{-1})$, time constant for the diffusion

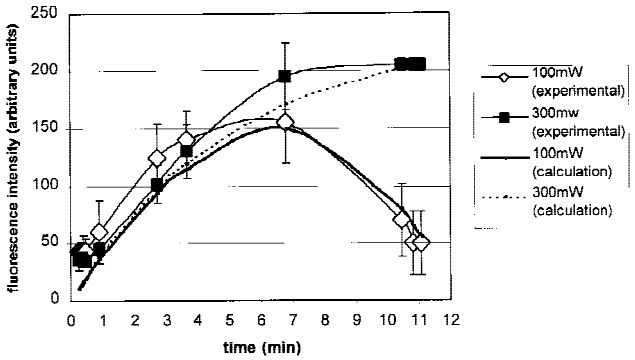


Fig. 5. Fluorescence intensity curves at the center of the laser spots for power 100 mW and 300 mW. For power 100 mW, the following equation was traced: $I_{center} = I_{max}$. Fitting parameters were: $I_{max} = 230$, $\alpha = 0.2$, $\beta = 0.46$ and $C = 0.92$ (correlation coefficient $r^2 = 0.992$). For power 300 mW, the following equation was traced: $I_{center} = I_{max}(1 - e^{-\alpha t})$. Fitting parameters were: $I_{max} = 230$, $\alpha = 0.2$ (correlation coefficient $r^2 = 0.992$).

of fluorescein from the periphery to the center of the laser spot, $\beta = 0.46\ (s^{-1})$, time constant for the disappearance of fluorescence from the measurement field. $I_{max} = 230$, $C = 0.92$, fitting parameter. For this case, Figure 5 shows a very good agreement between experimental data and mathematical modeling (correlation coefficient $r^2 = 0.994$).

In the second one, power is equal to 300 ± 20 mW. In this case, the tissue is damaged. Histology shows that both the neural retina and the choroid are involved in the coagulation process. Coagulation necrosis of the blood within the choriocapillaries and within some of the larger choroidal vessels are disclosed. Consequently, the clearance process does not exist any more. Only term A of equation 1 is used. The same parameters are used in equation 1: $\alpha = 0.2$ and $I_{max} = 230$.

For this case, calculation shows also a very good agreement between experimental data and mathematical modeling (correlation coefficient $r^2 = 0.992$).

DISCUSSION

The quantification of tissue response to laser photocoagulation is of critical importance particularly when using infrared diode laser. The effects of an 810 nm diode laser are less predictable since its longer wavelength contributes to an increased penetration of the laser light. Consequently, some difficulties exist in clinical practice for the control of the intensity of the retinal burn. Although fluorescein angiography is commonly used, only a few studies report the use of this method to detect or to quantify laser induced retinal lesions. Wiedenthal has used fluorescein angiography to study fundus coagulation of the rabbit [4]. Similarly, Baurmann has shown that fluorescein angiography gave effective information for estimating the applied laser effect [5]. However, their works were limited to the possible significance and applications of this method. More recently, Borland has shown that fluorescein angiography detects laser-induced retinal lesions at radiant exposure levels between those that produce ophthalmoscopically visible changes and those that can only be detected by microscopy [15]. In general, angiography could detect retinal changes at total intraocular energy levels about 60% below those required to induce changes detectable by ophthalmoscopy. For all of Borland's experimental conditions, fluorescein angiography has proved to be more sensitive and reliable than ophthalmoscopy. Similarly, the interest of fluorescein angiography was demonstrated by Hayashi [16]. He observed that the angiographic findings closely matched those observed by scanning electron microscopy. This finding is very important regarding the measurement of the extent of laser-induced coagulation and the development of techniques for feedback control of photocoagulation. Fluorescein could be more powerful using fluorescein angiography than reflectance alone [17,18]. This observation is confirmed by our study. For low power (100 ± 20 mW), no whitening was seen by ophthalmoscopy whereas a fluorescence spot was observed for up to 7 minutes after fluorescein injection. The histologic findings confirmed that fluorescein could escape the vascular compartment due to the loss of the vascular integrity. The surrounding tissue might retain fluorescein at an extent depending on the physicochemical modification of the interstitial matrix.

The most important aspect of our study is the possibility to quantify sequential changes in the retina and the choroid following transpupillary diode laser irradiation. Staining of laser-induced lesions appeared to be very dependent on laser energy. As soon as the power was superior to 100 ± 20 mW, the lesions exhibited a fluorescent ring. In that case, the fluence rate was sufficient to reach temperatures leading to a loss of structural integrity of cells. Thus, sodium fluorescein was able to penetrate the blood retinal barrier by both inter- and intracellular routes. It must be noted that the fluorescence intensity evolution at

the borders was independent of the laser dosage since the fluorescence ring was always obtained for the same temperature gradients. This particular ability of sodium fluorescein to stain laser-induced lesions was also demonstrated by Piccolino [19]. This author observed that with sodium fluorescein the dye staining in the photocoagulated areas was most marked between 5 and 10 minutes. A fluorescence plateau appeared for a threshold light dose above 200 ± 20 mW. This value was in accordance with that obtained by Cho [20]. His study of histopathologic and ultrastructural findings of photocoagulation lesions produced by transpupillary diode laser in the rabbit retina show that grade 3 photocoagulations are obtained for 200 ± 20 mW light doses.

In our study, an irreversible damage of the RPE (retinal pigment epithelium) cells was obtained as soon as the fluorescence at the center of the laser spot remained stable. Hayashi used fluorescein angiography to observe cellular changes in the retinal pigment epithelium after laser photocoagulation has confirmed that in the first 5 minutes, the damaged RPE cells appeared as negative fluorescent spots with intense fluorescent margins [16]. The histologic study showed that most of these cells had a condensed or coagulated appearance throughout their cytoplasm, which indicated both a more extensive passage of thermal transients and a more prolonged temperature elevation. Thus, in the first minutes, the entire central plaque represented a diffusion barrier to fluorescein. The loss of fluorescein from this central region was probably also enhanced by nonperfusion of vessels of the choriocapillaris within this area resulting from a thermally induced occlusive response. Consequently, the latent infilling of the central zone during angiography arose from diffusion of NaF from the annuli into the central region of coagulated photoreceptors. NaF remains in this tissue and could be bound to unknown species (extracellular or cellular substances released after cell breakdown). Another explanation could be that the persistent fluorescence of the laser lesions might be explained by the fact that damaged cells in the retina and the choroid would constitute sites of enhanced attraction for the fluorescein, due to the fact that these swollen cells were impregnated with fluorescein. If more investigations are needed in order to understand the staining process, it appears that the mathematical modeling applied in this study could be a good representation of the mechanisms underlying the fluorescence kinetics of both damaged and undamaged tissue. Our results confirm that the assumption that the quantification of laser-induced damage via measurement of fluorescence intensity is self-consistent.

In conclusion, this study shows that quantitative assessment of the laser-induced damage to the retina is feasible using fluorescence imaging. The quantification of fluorescence staining in terms of both intensity and time can contribute to a better quantification of laser-induced damage and can be correlated to histologic observations. So, a photocoagulation grading can be obtained instead of retina discoloration, which is usually the parameter used by ophthalmologists. Fluorescein angiography has long been useful for the evaluation of vascular disorders, it may be considered for the quantification of diode laser-induced damage related to the treatment of choroidal neovascular membranes or diabetic macular edema. At last, since laser damage may mimic naturally occurring pathology, this method should also be considered to quantify different types of lesions at an early stage. The data analysis procedure can be easily automated to facilitate the quantification of the laser-induced damage.

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